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Chin Med Sci J
June 1999

Vol. 14, No. 2
P. 102—106

CHINESE
MEDICAL SCIENCES
JOURNAL

THE RELATIONSHIP BETWEEN EXPRESSIONS OF N-myc AND c-myc ONCOGENES IN NEUROBLASTOMA: AN *IN SITU* HYBRIDIZATION AND IMMUNOCYTOCHEMICAL STUDY*

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Key words neuroblastoma; N-myc; c-myc

N-myc gene amplification is the most characteristic feature of neuroblastoma. c-myc oncogene, another member of myc gene family, plays an important role in cell proliferation and differentiation. Both of them may contribute to tumorigenesis of neuroblastoma. In this study we use the *in situ* hybridization and immunocytochemical methods to test the frequencies of N-myc and c-myc expressions in 20 cases of human neuroblastoma at mRNA and protein levels. The positive rates of the expression of N-myc are 90% and 100% detected by *in situ* hybridization and immunocytochemical methods respectively. The positive rates of c-myc are 80% and 85% respectively. Sixty percent of the 20 specimens tested by *in situ* hybridization and 55% by immunocytochemistry show an inverse relationship between the expressions of these two oncogenes and this may indicate that there are different gene expression controlling mechanisms in different cases.

INTRODUCTION

Neuroblastoma originates from the sympathoadrenal lineage of the neural crest and it is the most common extra-cranial tumor of childhood(1). Despite distinct improvement in treatment of this tumor, children with advanced-stage disease still have a poor prognosis. Recent cytogenetic and molecular biological studies have

Received for publication Feb. 16, 1998.
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* This investigation was supported by Grants from National Committee of Education and Grants for Outstanding Yong Scientist.

led to a better understanding of this tumor. N-myc gene amplification and over-expression are the most characteristic feature of neuroblastoma, which closely correlate with clinical outcome (2). c-myc oncogene is another member of the myc gene family, which plays an important role in cell proliferation and differentiation, and it can also induce cell apoptosis (3). Both N-myc and c-myc oncogenes may contribute to tumorigenesis of neuroblastoma. In this study, we use both *in situ* hybridization and immunocytochemical methods to detect the expressions of these two oncogenes in neuroblastoma at mRNA and protein levels, and try to find some clues of the relationship between the expressions of these two oncogenes.

MATERIALS AND METHODS

Tissue preparation. The formalin-fixed, paraffin-embedded surgically resected neuroblastoma tissues obtained from 20 patients were collected from the Department of Pathology, Beijing Children's Hospital. Tissues were cut into 5 μm thick sections and mounted on poly-L-lysine-coated slides.

Cell lines. The cell line IMR-32/NGFR, which was derived from human neuroblastoma cell line IMR-32 by transfecting with recombinant NGFR retroviral vector, was used as positive control for N-myc, since it had been proved to have over-expression of N-myc oncogene (4). Human pancreatic carcinoma cell line PC-2 was established in our department and served as positive control for c-myc, since it had a high expression of c-myc (5).

Probes. Plasmids containing N-myc and c-myc cDNA fragments (1.0kb both) were kindly provided by Dr. A. H. Ross from USA. The probes were labeled by the random primed labeling method using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim).

In situ hybridization (6). Tissue sections were deparaffinized in xylene, rehydrated through a graded ethanol, rinsed in PBS and treated with proteinase K (100μg/ml, 30 min at 37℃). The slides were then refixed in 4% paraformaldehyde solution for 5 min, rinsed in PBS, and covered with hybridization solution containing 1 ng/μl heat-denatured probe, 50% formamide, 0.5% SDS (sodium dodecyl sulphate), 100 μg/ml sheared salmon sperm DNA, 6×SSC (1×SSC was composed of 150 mmol/L NaCl and 15 mmol/L sodium citrate) and 5×Denhardt's solution. The slides were incubated overnight at 42℃. After hybridization, the slides were stringently washed as follows: 0.1% SDS, 2×SSC twice for 10 min at 42℃; 50% formamide, 2×SSC for 10 min at room temperature, twice; 50% formamide, 0.5×SSC for 10 min at room temperature, twice; 0.2×SSC for 5 min at room temperature; 0.1×SSC for 2 min at room temperature. The slides were then visualized using anti-digoxigenin-alkline phosphatase conjugate and NBT (nitro-blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrates according to the instruction of the kit.

Antibodies. Rabbit anti-human N-myc polyclonal antibody corresponding to amino acid 445-463 mapping at the carboxy terminus of human N-myc oncoprotein was purchased from santa Cruz Inc., USA. Mouse anti-human c-myc monoclonal antibody corresponding to amino acid 408-420 of human c-myc oncoprotein was purchased from Novo Inc., USA.

Immunocytochemistry. Tissue sections were deparaffinized and rehydrated, then covered with endogeneous peroxidase blocking solution for 10 min, rinsed in PBS, 10% non-immune serum for 20 min, rinsed in PBS, then the primary antibody was applied (the two antibodies were all diluted to 1:50), and incubated overnight at 4℃. After rinsing in PBS thoroughly, the biotinylated second antibody was added and left for 10 min. Then the slides were rinsed in PBS, streptoavidin-peroxidase conjugate solution was applied to the slides and left for 10 min, rinsed in PBS, then visualized with a solution containing 0.05% 3, 3'-diaminobenzidine and 0.01% H_2O_2, sections can also be counterstained with hematoxylin.

RESULTS

General clinical and histopathological features. The mean age of these 20 patients is 2.2 years, ranging from 1 month to 5 years. The ratio of male and female is 1.86:1 (13 males and 7 females). The sites of these 20 tumors are as follows: 8 in retroperitoneal region, 6 in adrenal glands, 2 in mediastinum, 1 in anterial region of sacrum and 3 from lymph node metastasis. Histologically, most tumors are composed of small round cells, with deep stained nuclei and scant cytoplasm, arranging in solid sheets, and hyperplastic blood vessels are prominent between the sheets. Among these tumors, Hower-Wright rosettes are found in 8 cases, and neurofibrile network is prominent in 5 cases. In 3 cases, the foci of ganglioneuroma are found. In another one, the tumor is composed mainly of ganglioneuroma and only small foci of neuroblastoma is found. The latter four patients are older than 4 years.

Expressions of N-myc and c-myc oncogenes. Results of the expressions of N-myc and c-myc oncogenes detected by the two methods are shown in Table 1.

The positive signals detected by *in situ* hybridization are shown as violet or blue deposits located mainly

Table 1. Expressions of N-myc and c-myc oncogenes in neuroblastoma by in situ hybridization (ISH) and immunocytochemical (ICC) method

Case	N-myc		c-myc	
	ISH	ICC	ISH	ICC
1	++	±	±	−
2	+±+	++	±	±
3	−	±	+++	++
4	±	±	+++	++
5	+	−	+	±
6	−	±	+±+	+++
7	+	±	−	−
8	++	+	+	±
9	+++	++	+++	−+
10	+++	++	−	±
11	±	±	+++	++
12	++	+	−	+
13	++	+	++	+
14	+++	−+	++	+
15	++	+−+	±	++
16	++	+±	±	−
17	±	±	+	++
18	+	±	−	−
19	+±−	++	++	+
20	++	+	+	++

in nuclei and perinuclear regions due to scanty cytoplasm of neuroblastoma cells (Fig 1); the positive signals of immunocytochemical detection are brown deposits located mainly in nuclei. According to the proportion of positive cells in the tumors, the detecting results are divided as follows: ± (positive cells fewer than 25%), + (25%~50%), ++ (50%~75%), +++ (more than 75%). The expression rates of N-myc and c-myc are 90% and 80% respectively as detected by *in situ* hybridization method. The immunocytochemical method shows the similar results, i. e. expression rates of N-myc and c-myc are 100% and 85% respectively. There is no significant difference between the positive rates obtained by the two methods.

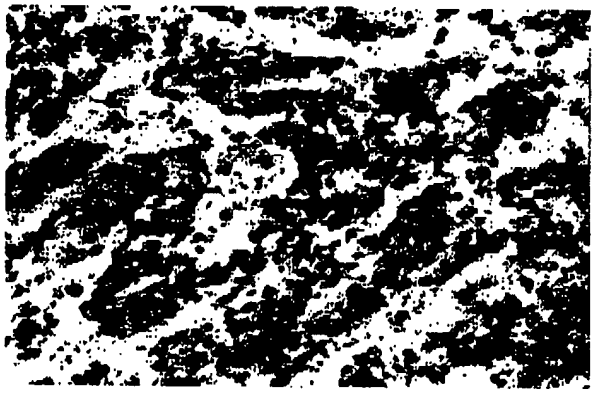

Fig 1. The positive signals of N-myc oncogene by *in situ* hybridization method are violet-blue deposits locating mainly in nuclei and perinuclear regions.

The relationship between the expressions of the two oncogenes. It was found by *in situ* hybridization method that 8 out of 20 cases of neuroblastoma which have relatively strong N-myc expressions (++~+++) have no or only weak c-myc expressions (+~−) (Fig 2A, B); whereas 4 cases with strong c-myc expressions (++~+++) have no or only very weak N-myc expressions (Fig 3A, B). Immunocytochemical method also gives the similar results. Only 20% of the 20 cases detected by *in situ* hybridization and 10% detected by immunocytochemical method show both strong expressions (±++~+++) of the two oncogenes. This inverse relationship between the expressions of the two oncogenes is shown in Table 2 and Table 3.

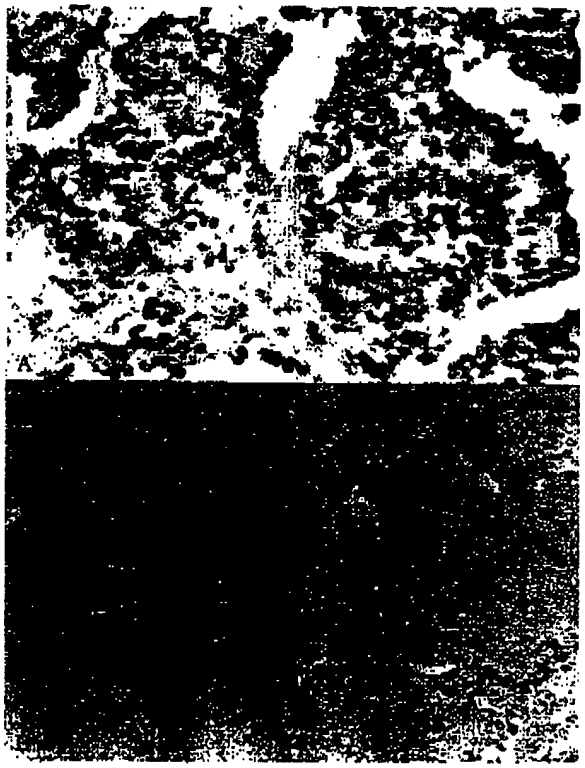

Fig 2. Neuroblastoma of case 2 shows strong N-myc expression (A) but very weak c-myc expression (B). (*in situ* hybridization)

DISCUSSION

Several genetic features which are the characteristics of neuroblastoma have been identified, these include

Fig 3. Neuroblastoma of case 4 shows strong c-myc expression (A) but very weak N-myc expression (B). (*in situ* hybridization)

Table 2. Comparison between the strength of expressions of the two oncogenes as detected by in situ hybridization method

		c-myc		
		++~+++	−~+	Total
N-myc	++~+++	4	8	12
	−~+	4	4	8
	Total	8	12	20

Table 3. Comparison between the strength of expressions of the two oncogenes as detected by immunocytochemical method

		c-myc		
		++~+++	−~+	Total
N-myc	++~+++	2	5	7
	−~+	6	7	13
	Total	8	12	20

hyperdiploidy, deletion of 1 p, and amplification of N-myc oncogene (7—9). The amplification of N-myc could be the most important feature in neuroblastoma since the tumors with amplified N-myc usually are associated with rapid tumor progression and a poor outcome. A general correlation between N-myc copy number and expression has been shown, but it has been shown that a substantial number of tumors without N-myc amplification also overexpress this gene (10-12). In this study, it was found that there were quite high rates of expression of N-myc oncogene in these cases, i. e. 90% by *in situ* hybridization method and 100% by immunocytochemical method. This may indicate that over-expression of N-myc may play an important role other than gene amplification, since only about 25% to 30% of the children with neuroblastoma have N-myc amplification in their tumors. Meanwhile it was also found there was a high expression rate of c-myc oncogene in these cases by *in situ* hybridization and immunocytochemical methods. Along with the result of N-myc expression, we believe that the two oncogenes may contribute to the tumorigenesis of neuroblastoma. Although N-myc and c-myc can co-express in the same tumor, it is interesting to note that 60% of the 20 cases show an inverse relationship between the expressions of the two oncogenes when we compare the strength of expression of them. The cases with strong expression of N-myc usually have very weak expression of c-myc, and *vice versa*. Studies on neuroblastoma-derived cell lines also provide similar results (13, 14), but the mechanism is poorly understood so far, it may represent the important event in gene regulation process controlling cell growth and differentiation. Further studies are needed to clearify the relationship between the expressions of these two oncogenes.

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Preparation of Recombinant α2 Antigen of *M. Leprae* in *E. Coli* and the Application for Sero-diagnosis of Leprosy

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Leprosy is remain a major public health problem in some developing countries. Because *M. leprae* cannot be grown in vitro, purified protein of native antigen is unavailable. An antigen is a prominent target of the humoral and cellular immune responses against mycobacteria and may be useful to the development of novel vaccines and immunodiagnostic reagents. For the development of novel immunodiagnostic reagents, the recombinant α2 antigen of *M. leprae* was prepared using the molecular biologic tools and the recombinant DNA expression technology.

Screening of the *M. leprae* expression library was performed by the plaque hybridization technique. Nucleotide sequences were determined by dideoxy termination method using A. L. F. florescent auto DNA sequencer. pMALc-RI was used as an expression vector. The antibody titer of anti-recombinant *M. leprae* α2 antigen in leprosy was examined by enzyme-linked immunosorbent assay (ELISA).

The results are: (1) By screening 10 000 plaques of *M. leprae* genomic library, two clones of α2 gene could be obtained. One of the two clones carrying the α2 gene was selected and digested by appropriate restriction enzymes, then were cloned into pUC18 or 19 sequencing vector. Determination of DNA sequence was performed by dideoxy termination method using A. L. F. florescent auto DNA sequencer. The complete nucleotide sequence date of α2 antigen gene was assigned in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession number D43841. (2) 200ml of LB medium were inoculated with over night culture of *E. Coli* carring *plasmid DNA* which can express MBP-*M. leprae* α antigen fusion protein. Cells were grown to mid log phase and the expression of MBP-*M. leprae* α antigen fusion protein in the sonic extracts was absorbed to amylose resin followed by elution with 10mmol/L maltose. More than 10mg of fusion protein were obtained from the 200ml of culture. (3) The sensitivity of α2- ELISA and PGL-I-ELISA determined on the sera of L leprosy was 92% and 94%, respectively. The corresponding results for B and T leprosy patients were 58% and 68%, 38% and 30%, respectively.

Although *M. leprae* was the first identified bacterial pathogen of man, basically biochemical, immunological, diagnostic and therapeutic investigations have been severely limited because it remains one of the few human pathogens that have not been cultured in vitro. The construction of *M. leprae* recombinant DNA genomic library could provide a source of gene encoding proteins relevant for such studies, and the molecular cloning, expression of M. leprae DNA in a suitable host vector system offers a new way of obtaining large amount of well-characterized antigens. The results of this study suggested that the recombinant α2 antigen could be used as a new specific antigen for sero-diagnosis of leprosy, especially the lepromatous leprosy.

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1: Chin Med Sci J. 1999 Jun;14(2):102-6.

The relationship between expressions of N-myc and c-myc oncogenes in neuroblastoma: an in situ hybridization and immunocytochemical study.

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N-myc gene amplification is the most characteristic feature of neuroblastoma. c-myc oncogene, another member of myc gene family, plays an important role in cell proliferation and differentiation. Both of them may contribute to tumorigenesis of neuroblastoma. In this study we use the in situ hybridization and immunocytochemical methods to test the frequencies of N-myc and c-myc expressions in 20 cases of human neuroblastoma at mRNA and protein levels. The positive rates of the expression of N-myc are 90% and 100% detected by in situ hybridization and immunocytochemical methods respectively. The positive rates of c-myc are 80% and 85% respectively. Sixty percent of the 20 specimens tested by in situ hybridization and 55% by immunocytochemistry show an inverse relationship between the expressions of these two oncogenes and this may indicate that there are different gene expression controlling mechanisms in different cases.

PMID: 12901618 [PubMed - indexed for MEDLINE]